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Detection of AKR MuLV-specific RNA in AKR mouse cells by in situ hybridization

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ABSTRACT

Conditions for the detection of complex RNA sequences by in situ hybridization have been investigated by using a single-stranded ^{3}H-cDNA probe complementary to the AKR MuLV genome and in vitro cultured AKR mouse cells which spontaneously produce AKR MuLV. It is shown that fixation with glutaraldehyde at low concentration allows cellular RNA to be sufficiently well retained during the annealing process and that stringent conditions in situ can be maintained by means of formamide. Some conditions which promote atypical and non-specific binding of the probe have been identified.

INTRODUCTION

Detection of specific RNA sequences in individual cells by in situ hybridization with complementary radioactive probes and autoradiography has been reported by several investigators (1,2,3,4,5). This technique is suited for studies of normal or abnormal development of small groups of cells that are difficult to investigate by bulk methods, or in cases where there may be asynchrony of expression of a particular parameter of interest within a given population of cells. In cells infected or transformed by viruses, the great excess of viral transcripts relative to DNA proviral sequences offers a decisive advantage in sensitivity of their detection (3).

Thus far there have been no systematic studies of in situ hybridization to RNA and published work has dealt mainly with the methods used to detect cellular DNA fractions. Because of the difference in lability of DNA and RNA molecules within cells, however, methods that are optional for DNA will not necessarily apply in the case of RNA. In view of this we have investigated extensively the procedure for in situ detection of cellular transcripts. Particular attention was paid to the fixation of target molecules, the specificity of the hybridization process and the detection of potential artefacts. The cells used were AKR mouse embryonic

cells (AKR/ac) in which murine leukaemia virus, AKR MuLV, is spontaneously induced (6,7). Virus-specific RNA was detected with a ^{3}H-cDNA probe of high specific activity complementary to the AKR MuLV genome, and in vitro and in situ hybridization were compared under similar annealing conditions.

MATERIAL AND METHODS

Cell culturing: An established cell line (AKR/ac) derived from AKR inbred mice(6,7)was used from passage 70 to 110. The cells were maintained by serial passage in McCoy's modified 5a medium supplemented with 10% foetal calf serum and antibiotics. They were routinely tested for PPLO, yeast and bacterial contamination as well as for the presence of mature C-type particles and budding virions in electron microscope (8). Chick embryo fibroblasts obtained from 11 days - embryonated eggs were grown in Macpherson-Stoker modified Eagle minimal essential medium containing 10% tryptose phosphate broth, 10% calf serum and antibiotics.

Cytological preparations were obtained by seeding the cells in Petri dishes (10cm diameter) containing sterile glass slides, at a concentration of $2x10^{6}$ cells per dish, 24hr prior to utilisation.

Labelling of cellular RNA: Cell preparations were incubated for 1hr 30 in fresh culture medium containing 15µCi/ml of 5-^{3}H-uridine (sp.ac. 29Ci/mmol), washed with Hank's solution, and incubated for 1hr 30 in normal medium. After incubation preparations were washed in Dulbecco A and fixed. Fixation was carried out at room temperature, for 20 min, followed by dehydration in 50, 70, 90 and 100% ethanol. The following fixatives were used: acetone, glacial acetic acid, ethanol/acetic acid 3:1 (v:v) or glutaraldehyde (EM scope Laboratory) at various concentrations in 0.1M Na cacodylate pH 7.2. Fixed preparations were either rinsed for 15 min in cold acetate buffer (0.3M NaCl; 0.01M Na acetate pH 5.0), dehydrated in ethanol and air-dried, or treated as stated in text, then dehydrated, air-dried, and autoradiographed as described below.

"In situ" hybridization: cytological preparations were removed from tissue culture Petri dishes, washed in five consecutive baths of Dulbecco A (250ml), immersed in glutaraldehyde for 20 min, at room temperature, and dehydrated in an ethanol series. Unless otherwise mentioned in text, glutaraldehyde was prepared at a final concentration of 0.1% in cacodylate buffer as stated above. Dehydrated preparations were air-dried for a few minutes and used without delay. Hybridization was carried out in

the acetate buffer already described, using 5µl of annealing solution placed under clean sterile coverslips (18x18mm) sealed with rubber solution. Preparations were incubated on a steel tray floating in an equilibrated covered water bath, and chilled on ice at the end of the hybridization period. Coverslips were then removed in cold acetate buffer to prevent drying. After rinsing in the same buffer slides were immersed for 15 min in S1 nuclease buffer (9) without glycerol, at 4°C. S1 nuclease (Sigma) was prepared at 100U/ml in S1 buffer containing 90µg/ml of denatured Micrococcus lysodeikticus DNA, and 20µl of this solution was applied under sealed coverslips (20x20mm). Digestion was for 1hr at 37°C. Slides were then washed for 1hr in 2xSSC at 55°C and for 6hr, or overnight, in cold acetate buffer, using a 3 litre stirred bath, and finally dehydrated in ethanol.

Autoradiography: Slides were exposed at 4°C to Ilford K2 emulsion diluted 1:1 with distilled water. Autoradiographs were developed in D19b (12 min, 4°C), fixed with Ilford Hypam diluted 1:4, for 4 min at 4°C, washed in tap water and stained with 2% Giemsa. Autoradiographic grains were counted under a light microscope in samples of randomly selected cells and intercellular background was deduced in the numerical data reported. 50 cells per point were counted in the case of uridine-labelled cells and 25 cells per point were counted in hybridized preparations.

Preparation of AKR MuLV 70S RNA: AKR MuLV was obtained from cultured AKR/ac cells (10). Viral 70S RNA was isolated from purified virions using the method of Waters et al. (10).

"In vitro" synthesis of AKR-V ^{3}H-cDNA: AKR-V cDNA was synthesized in an endogenous reverse transcriptase reaction using AKR MuLV purified virus, 0.01% NP40, 50mM Tris-HCl pH 8.0, 50mM NaCl, 5mM MnCl2, 5mM dithiothreitol, 1mM dATP, dCTP, dGTP, 4µM ^{3}H-TTP (50 Ci/mmol) and 50µg/ml actinomycin D. The reaction was carried out at 37°C for 5hr and followed by phenol extraction and purification on Sephadex G50 (11,12).

The calculated specific activity of ^{3}H-cDNA was $8x10^{7}$dpm/µg, and its length was 70-100 nucleotides by velocity sedimentation in an alkaline sucrose gradient (4).

96 to 98% of the probe was digestible by S1 nuclease (200U/ml of enzyme in conditions reported above).

^{3}H-cDNA (3,000 cpm) was annealed to 70S viral RNA (6.6µg/ml) in acetate buffer plus 0.05% SDS and $5x10^{-4}$M EDTA. Aliquots of 20µl of reaction mixture were covered with paraffin, boiled (11) and placed at 65°C.

Hybrids were analyzed by S1 nuclease digestion. It was found that 65% of the radioactivity became S1 nuclease resistant over a period of 6hr. Thus at least 65% of the ^{3}H-labelled probe is complementary to viral 60-70S RNA. This is in reasonable agreement with the values reported by other authors (11).

RESULTS

Fixation of RNA for "in situ" hybridization. A number of different fixatives which act as dehydrating agents, such as alcohol or acetone, or as additive agents, such as aldehyde (13) were compared. Preservation of cellular components in cells subjected to annealing conditions was monitored by light microscopy and the retention of autoradiographically detectable RNA was checked by prelabelling the cells with 5-^{3}H-uridine.

Cells fixed as described in Table 1 were subjected to the following alternative treatments designed to investigate the stability of cellular RNA under conditions used in in situ hybridization:

1) incubation at 40°C in a large excess of annealing solution, either 2xSSC or acetate buffer, for various periods of time;

2) incubation in 0.2N HCL for 20 min at room temperature, a treatment used in previous studies in order to remove proteins that might interfere with hybridization;

3) HCl incubation as in 2, followed by annealing buffers as in 1.

Cell morphology and stainability after incubation were noticeably poorer with dehydrating fixatives and loss or disruption of cytoplasm was frequently observed as reported by other authors (14). In contrast cells fixed with glutaraldehyde at a concentration as low as 0.1% were easily stained and exhibited a well preserved morphology (15). Preservation of

TABLE 1

Mean number of grains per cell in autoradiographs of AKR/ac cells labelled with 5-^{3}H-uridine and treated with 0.2N HCl for 20 min at 20°C.

Fixation	A untreated cells			B treated cells	% cells with no grains in treated cells
	total	cytoplasm	nucleus		
Acetic Acid	246	113	133	1	34
Acetone	259	124	135	1	46
Ethanol/acetic Ac.	362	166	196	0.2	56
Glutaraldehyde 0.1%	301	162	139	2.54	24

subcellular components in such cells after long-term incubation in acetate buffer was confirmed by electron microscopy (data not shown).

Treatment of fixed preparations with 0.2N HCl leads to a substantial loss of autoradiographically detectable RNA (Table 1). Labelled cells treated with acid partially regained their capacity to produce an autoradiograph when incubated in annealing buffer for short periods of time. This suggests that HCl removes RNA preferentially from the superficial layers of the cytoplasm leaving radioactive molecules in regions which initially lie beyond the range of the tritium disintegrations which produce an autoradiograph. Due to cell shrinkage, loss of masking substances or RNA mobilisation, such molecules are subsequently brought within the range of autoradiographic emulsion as a result of the annealing process.

In view of these findings we discontinued the use of 0.2N HCl and of dehydrating fixatives. The remaining experiments concentrated upon the investigation of glutaraldehyde fixation.

Since cross-linking of proteins by glutaraldehyde is likely to impede the access of probes in subsequent hybridization (16,17), it was necessary to determine optimal fixation conditions which would allow cellular RNA to be retained while at the same time permitting efficient hybridization in situ. It was found that such conditions could be achieved by using low concentrations of fixative. As shown in Table 2, the percentage of RNA which is retained after incubation depends, in this case, upon the annealing buffer in use and upon the duration of the annealing period.

"In situ" hybridization of AKR-V cDNA to glutaraldehyde fixed cells

A) Optimal fixation conditions. The concentration of aldehyde permitting optimal hybridization was determined by hybridizing AKR-V cDNA (1µg/ml in acetate buffer plus 21% formamide, 45°C, 4hr 30) to cells fixed with

TABLE 2

Percentage of grains in autoradiographs of AKR/ac cells labelled with 5-^{3}H-uridine and incubated in annealing solutions, at 40°C, for varying periods of time.

Concentration of glutaraldehyde (%)	Acetate buffer			2 x SSC		
	0 hr	3 hr	24 hr	0 hr	3 hr	24 hr
0.1	100	90	58	100	55	38
0.25	100	90	82	100	84	48
0.5	100	90	91			
1	100	79	77			

varying concentrations of fixative, and estimating the number of grains per cell after 7 days exposure. As shown in Table 3B, the mean values thus obtained show a regular pattern of variation, the maximum being reached at 0.05-0.1% glutaraldehyde. Identical results have been found by hybridizing ^{3}H-poly(U) to poly(A) sequences from AKR/ac cells (15).

B) The use of formamide. Hybridizing the probe at 60°C without formamide, in conditions otherwise similar to those reported above, led to a contrasting pattern in which the number of grains per cell was greater and did not appear to vary with the fixative concentration (Table 3A, 17 days exposure).

The above data suggested that hybridization to RNA in situ might be affected differently under conditions of equivalent stringency, at high or at low temperature, in the absence or in the presence of formamide. To investigate this point we constructed initial rate curves for AKR-V cDNA hybridized either to AKR-V 70S RNA in solution or to AKR/ac cells, over a range of equivalent stringency, with and without formamide.

AKR-V ^{3}H-cDNA (0.02µg/ml) was annealed to AKR-V 70S RNA (6.6µg/ml) in aqueous acetate buffer plus 0.05% SDS and 5×10^{-4}M EDTA. Aliquot samples of 20µl were covered with paraffin, boiled (11) and placed in water bath at various temperatures, for 3 hr, as reported on Fig. 1A. The reaction was stopped by diluting in ice cold buffer and hybrids were recovered after treatment with S1 nuclease. Under these conditions it was determined that less than 20% of the probe became S1 resistant at 65°C. In a parallel assay, AKR-V cDNA was annealed to 70S RNA at 45°C in the presence of increasing concentrations of formamide (Fig. 1B). Experimental conditions were identical to those mentioned above in all other respects.

TABLE 3

"In situ" hybridization of AKR-V cDNA to AKR/ac cells fixed with varying concentrations of glutaraldehyde: influence of temperature.

Concentration of glutaraldehyde (%)	Mean number of grains per cell at: 60°C (A)	45°C+21% formamide (B)
2.5	202	12
1	-	9
0.5	144	11
0.25	-	22
0.1	195	41
0.05	168	66
0.025	244	41

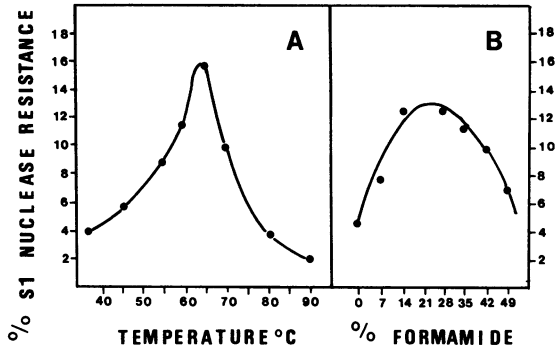

Fig. 1. Hybridization of AKR-V cDNA to 70S RNA in solution, at varying temperature without formamide (A) and in varying formamide concentration at constant temperature (B).

From Fig. 1A it can be seen that, in the hybridization initial rate experiment, the maximum S1 nuclease resistance in aqueous buffer occurs sharply at 64°C. From Fig. 1B, as expected, the maximum S1 resistance in formamide is lower and occurs over a broader range but under approximately similar conditions of stringency, since a temperature of 45°C with 17% formamide is approximately equivalent to a temperature of 65°C without formamide (18).

Whether the hybridization reaction in situ exhibits similar dependence upon temperature and formamide concentration was subsequently examined (Fig. 2). In Fig. 2A cells were incubated at 45°C with 1μg/ml of cDNA in acetate buffer plus increasing concentrations of formamide. In Fig. 2B cells were incubated at varying temperature with 1μg/ml of cDNA in aqueous buffer alone. In both cases hybridization was for 4hr 30 and exposure for 9 days.

Results indicate that the rate of the in situ reaction is also dependent upon temperature and organic solvent concentration. However it should be noted that, above a temperature of 53-55°C, the mean number of grains per cell obtained in aqueous buffer is higher than the number obtained at 45°C in formamide under conditions of equivalent stringency (18).

As control the probe was annealed to chick embryo cells which were treated and exposed in parallel with AKR/ac cells. The mean grain count per cell in three chick embryo preparations hybridized at 45°C with

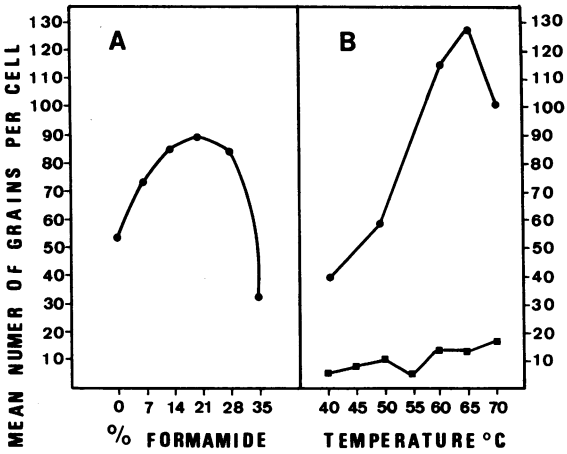

Fig. 2. "In situ" hybridization of AKR-V cDNA to cells fixed with 0.1% glutaraldehyde, in formamide (A) and at varying temperature (B). -●-●-: AKR/ac cells; -■-■-: chick embryo cells.

21% formamide was 18, 13 and 9 respectively. The mean grain count obtained at varying temperature is depicted in Fig. 2B which shows that intracellular background labelling, as determined on heterologous cells, slightly increases with temperature.

As discussed below, the increase in the yield of autoradiographic grains obtained in AKR/ac cells without formamide might partially reflect non-specific binding of the probe. For this reason, hybridization was subsequently carried out in formamide using the optimal concentration determined above.

C) Characterization of the reaction. In the experiments described below slides were fixed with 0.1% glutaraldehyde, annealed at 45°C in 21% formamide and exposed for 9 days.

The dependence of the reaction upon the input concentration of cDNA was shown by hybridizing the probe for 4hr 30 at concentrations stated in Table 4. In the same experiment intra-cellular background labelling was tested on chick cells using the probe at a concentration of 1µg/ml. It is not known whether the relationship between background labelling and the specific autoradiographic signals would be maintained at higher concentrations.

A time course for hybridization is depicted in Fig. 3 which shows

TABLE 4

Mean number of grains per cell after hybridization with AKR-V cDNA at varying concentrations.

Concentration of input ^{3}H-cDNA in μg/ml	Mean number of grains per cell AKR/ac	chick cells
1	38	10
0.5	28	-
0.25	6	-
0.12	4	-
0.06	2	-

that the maximum yield of hybrids is not achieved until at least 11hr at the input concentration of probe used here (1μg/ml). The mean grain count on chick cells, after hybridizing AKR-V cDNA under similar conditions, was 6 and 13 at 4hr and 11hr respectively.

Independent experiments with AKR/ac cells gave reasonably reproducible results. The mean number of grains per cell per day, at cDNA input concentration of 1μg/ml, was 6 and 10 after annealing for 15hr, and 3,4,6 and 10 after annealing for 4hr 30.

All cell hybridization data were based upon randomly selected cells and it is apparent that although all cells were labelled, the number of grains over cytoplasm and over nucleus varied widely from cell to cell in the same preparation. This component of variability was not effectively altered by counting larger numbers of cells (15).

Pretreatment with ribonuclease was attempted as a negative control. AKR/ac cells were fixed as above, treated for 60 min at 37°C with 100 to

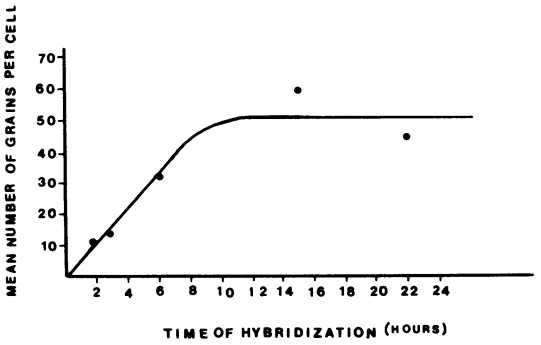

Fig. 3. Kinetics of "in situ" hybridization of AKR-V cDNA to AKR/ac cells.

800µg/ml of pancreatic RNase in acetate buffer, and hybridized for 15hr as in Fig. 3. The autoradiographic signal after 20 days exposure was stronger in the RNase pre-treated group than in the untreated controls and its intensity was dependent upon the RNase concentration used. By the use of other types of cells, such as chick embryo cells, it was shown that the effect brought about by pre-RNasing was non-specific.

DISCUSSION

In situ hybridization to RNA encounters a new set of problems which arise from the requirement to retain essentially labile target molecules in a suitable state for hybridization whilst, at the same time, avoiding artefacts arising from the simultaneous retention of other molecules. To this the method of fixation is of paramount importance.

We have examined the comparative effectiveness of different conventional fixatives under the subsequent annealing conditions used for *in situ* hybridization, and conclude that reasonably effective and comparatively the best preservation of cellular components, as judged by light and by electron microscopy, may be obtained by means of cross-linking fixation, as examplified by glutaraldehyde. For this reason we chose this fixative in subsequent studies.

By prelabelling cells with 5-^{3}H-uridine and using autoradiography we have investigated the stabilizing effect on RNA of low concentrations of glutaraldehyde. Since autoradiography detects only the labelled molecules whose disintegrations lie within range of the photographic emulsion (19) the method is not quantitative. It also cannot define whether there is differential loss of particular types of RNAs. Preliminary studies in which the radioactive RNAs released during the incubation of 0.1% glutaraldehyde-fixed cells in annealing solution were analysed by oligo-dT cellulose chromatography have indicated that there are significant losses of RNAs over an incubation period of 4 hours, approximately 20% of which is poly(A)+. The loss of both poly(A)+ and poly(A)- RNAs increased with time during an initial period of 9 hours, after which it levelled off. No fixative examined, which included ethanol/acetic acid and acetone, was particularly effective in preventing RNA loss under annealing conditions when tested by this method.

Glutaraldehyde at a concentration of 0.1% fixes cellular components as efficiently as higher concentrations during prolonged subsequent incubation in an appropriate buffer. This concentration is lower than

that usually used in electron microscopy and affects a compromise between the necessity of fixing RNA whilst avoiding an undue inhibitory effect on hybridization obtained at higher concentrations. This inhibitory effect conceivably may result from direct binding of aldehyde to RNA (16) and/or from steric hindrance generated by cross-linking of nucleoproteins.

In the present conditions of fixation higher temperatures appear to cause unspecific binding of probes. Thus the radioactivity unspecifically bound to heterologous chick cells, as measured by autoradiography, is higher at 65°C in aqueous buffer than it is at 45°C in formamide. When annealing the probe to AKR/ac cells in conditions of equivalent stringency, with or without formamide, the average grain count per cell found in the absence of formamide was increased more than expected from equivalent hybridization in solution, compared to controls hybridized in formamide at 45°C (compare Fig. 1 and 2). A similar effect was seen in comparisons made in Table 3. We do not know however the proportion of unspecific grains obtained at high temperature since we do not know the extent to which formamide might influence the rate of the reaction in situ.

When using the present conditions of fixation it is clearly important to keep the temperature of annealing low and to maintain stringent conditions by means of formamide. The optimal concentration of this solvent should therefore be experimentally determined. In situ determination is preferable since data from Figs. 1 and 2 indicate that, in the present conditions, the stability of hybrids in formamide is slightly lower in situ than it is in solution.

Because cells contain relatively minute amounts of RNA, cDNA must be in excess to drive the reaction (20) and high concentrations of probe are required in order to obtain a significant number of grains within short periods of time. It is also probable that, due to steric hindrance, the cellular RNA is not freely accessible to the probe.

At high input of probe, as expected for hybridization of heteropolymeric sequences, the rate of the reaction, although increased, is still slow compared with hybridization of tritiated poly(U) to poly(A) sequences from AKR/ac cells which shows very fast kinetics (15). The rate of the reaction in situ is therefore consistent with probe complexity.

Due to partial loss of RNA, uncertain efficiency of autoradiography and other unknown factors affecting the yield of hybrids, estimates of sensitivity of in situ hybridization to cellular RNA are not feasible. Furthermore, because of RNA mobility and the physical limitations to

tritium autoradiography (19) caution is necessary in interpreting the probable origin and location of the RNA that is detected.

In addition, we have identified a number of factors which promote artefactual binding of radioactive probes. Among such factors, the hydrolysis, or the release, of cellular components, at any stage of the hybridization process, appears important.

Acid hydrolysis with 0.2N HCl causes a substantial loss of cellular RNA. Furthermore, in separate experiments using radioactive poly(U) to detect poly(A) sequences in situ, a high background of autoradiographic grains was obtained following HCl treatment and immediate annealing of the probe (15). For these reasons, the use of HCl cannot be recommended. Moreover, further deproteinization of the fixed cells seems unlikely to be necessary, since it has already been shown that proteins in association with RNA may not prevent hybridization (21).

Pretreatment with RNAse was also found to cause unspecific binding of probes. A similar effect was originally described by Gillespie and Spiegelman in their study of DNA/RNA hybridization on filters (22). Previous investigations (3,4) have not encountered such an increased background in situ however the conditions they used differed in many other respects from those we describe here.

Non-specific labelling can also be generated by using high concentrations of S1 nuclease in the presence of low concentrations of denatured single-stranded DNA to digest unhybridized probes after annealing (data not shown).

The mechanism through which probes are unspecifically bound to hydrolysed cellular components is still unclear. Binding to RNase which is a basic protein (22), mechanical trapping by disrupted cell structures or binding due to electrical charges whose appearance may follow acid and enzymatic hydrolysis could be implicated in this process.

The results reported here, together with additional evidence drawn from hybridization of tritiated poly(U) to the same cell strain (15) indicate, however, that the origin of the autoradiographic grains obtained in the present conditions can be ascribed to a phenomenon of specific annealing though a low level of background labelling is still observed. With suitable precautions therefore this method is likely to be valuable in many studies dealing with regulation of the expression of cellular and viral functions.

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